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(54) **Endothelin receptors.**

(57) The amino acid sequence of an endothelin receptor is now analyzed. A DNA sequence encoding the receptor, a recombinant expression vector containing the DNA sequence, a process for production of the receptor, a protein composition containing the receptor and an agent for detecting the endothelin are disclosed.

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The DNA sequence of the present invention can be integrated into a expression vector to obtain a recombinant expression vector. Therefore, the present invention further provides a process for production of a mammalian endothelin receptor or an analogue thereof, which comprises inserting the recombinant expression vector into a host cell and culturing the cell under conditions promoting expression.

5 The invention furthermore provides a protein composition containing a biologically active mammalian endothelin receptor or an analogue thereof which is produced as mentioned above.

The obtained protein composition containing a biologically active mammalian endothelin receptor or an analogue thereof is effective in an assay of the mammalian endothelin. The composition is also available in preparation of an antibody to the endothelin receptor. The antibody can be used in diagnosis.

10 It is apparent from the above-described biological activities of endothelin and the receptor thereof that an agent containing an antibody to the endothelin receptor is effective in diagnosis of hypertension or hypotension.

#### BRIEF DESCRIPTION OF THE DRAWINGS

15 Figs. 1A and 1B show the amino acid sequence of the endothelin receptor.

Figs. 2A to 2C show the DNA sequence and the deduced amino acid sequence of the endothelin receptor.

Fig. 3 schematically illustrates pCDM8.

20 Fig. 4 is a graph showing a biological activity of the endothelin receptor with respect to production of inositol phosphates.

Fig. 5 is a graph showing a biological activity of the endothelin receptor with respect to increase of calcium concentration in cytoplasm.

Fig. 6 is a graph showing a biological activity of the endothelin receptor with respect to the endothelin binding characteristics.

Fig. 7 schematically illustrates an expression plasmid of the endothelin receptor.

#### DETAILED DESCRIPTION OF THE INVENTION

30 In the present specification, the term "endothelin receptor" means proteins which are capable of binding endothelin molecules and, in their native configuration as mammalian plasma membrane proteins, presumably play a role in transducing the signal provided by endothelin to a cell. In the specification, the term includes analogs of native proteins with endothelin-binding or signal transducing activity.

35 The term "subtype of endothelin receptor" means molecules of endothelin receptor which show different pharmacological potency rank orders, namely different affinities or selectivities for isopeptides of endothelin or sarafotoxin.

The term "substantially (or essentially)" used in the expression "a DNA sequence substantially encoding a mammalian endothelin receptor" or the like means that a particular subject sequence, for example, a mutant sequence, varies from a reference sequence by one or more substitutions, deletions, or additions, the net effect of which does not result in an adverse functional dissimilarity between reference and subject sequences. In more detail, a sequence having at least 30 % similarity is considered to be substantially identical. The similarity preferably is at least 50 %, and more preferably is at least 80 %. For purposes of determining similarity, truncation or internal deletions of the reference sequence should be disregarded. Sequences having lesser degrees of similarity, comparable biological activity, and equivalent expression characteristics are considered to be substantial (or essential) equivalents.

45 The term "biologically active" used as a characteristic of endothelin receptors means either that a particular molecule has sufficient amino acid sequence similarity with the embodiments of the present invention, or that a particular molecule has sufficient amino acid sequences similarity to be capable of transmitting an endothelin stimulus to a cell as a component of a hybrid receptor construct. In more detail, the affinity (dissociation constant) of a particular molecule for endothelin-1, 2 or 3 is not more than 1  $\mu$ M. In the present invention, the affinity preferably is not more than 0.1  $\mu$ M, and more preferably is not more than 0.01  $\mu$ M.

The term "biologically active" also means that a particular molecule has a function of accelerating production of inositol-1,4,5-triphosphate to increase calcium ion concentration in cytoplasm.

55 The term "DNA sequence" means a DNA polymer, in the form of a separate fragment or as a component of a larger DNA construct. The DNA construct is derived from DNA isolated at least once in substantially pure form (free of contaminating endogenous materials) and in a quantity or concentration enabling identification, manipulation, and recovery of the sequence and its component nucleotides se-

quences by standard biochemical methods, for example, using a cloning vector. The DNA sequences are preferably provided in the form of an open reading frame uninterrupted by internal nontranslated sequences, or introns, which are typically present in eukaryotic genes. However, genomic DNA containing the relevant sequences can also be used. Sequences of non-translated DNA may be present 5' or 3' from the open reading frame. The non-translated DNA does not interfere with manipulation or expression of the coding regions.

The term "recombinant expression vector" means a plasmid comprising a transcriptional unit. The unit comprises (a) a genetic element or elements having a regulatory role in gene expression, for example, promoters or enhancers, (b) a structural or coding sequence which is transcribed into mRNA and translated into protein, and (c) appropriate transcription and translation initiation and termination sequences. Structural elements used in yeast expression systems preferably include a leader sequence enabling extracellular secretion of translated protein by a host cell. In the case that a recombinant protein is expressed without a leader or transport sequence, it may include an N-terminal methionine residue. This residue may optionally be cleaved from the expressed recombinant protein to provide a final product.

Next, isolation of cDNA encoding the endothelin receptor and determination of the DNA sequence are described below.

A cDNA library was prepared by a reverse transcription of poly(A)<sup>+</sup> RNA, which was isolated from rat lung. The DNA sequence encoding the rat endothelin receptor was isolated from the cDNA library. The library was screened by direct expression of mRNA from DNA fragments accumulated in monkey COS-7 cells using a mammalian expression vector (pCDM8). The vector contains regulatory sequences derived from SV40, polyoma virus and cytomegalovirus. The cells expressing a biologically active endothelin receptor was identified by incubating transfected COS-7 cells in a culture containing [125I-Tyr13]endothelin-1, washing the cells to remove the free labelled endothelin-1 from the cells, and placing an X-ray film on monolayers of the cells to detect binding of endothelin-1. The detected transfected cells are recognized as black spots on the film.

According to the above-mentioned method, about 20,000 cDNA clones were screened in about 40 pools to detect a black spot showing binding of endothelin-1 by an assay with respect to one pool of transfected cells. Lyophilized bacteria derived from the positive pool were cultivated in a medium. With respect to a single colony formed on an agar medium by the bacteria, screening was repeated to identify a clone which synthesizes a surface protein having a detectable endothelin-1 binding characteristic. The clone was isolated, and the sequence of insertion fragments was analyzed to determine the cDNA sequence of the rat endothelin receptor.

COS-7 cells were transfected with isolated cDNA clone to express the gene. As a result, the cells obtained a specific binding activity to endothelin-1, 2 and 3 and sarafotoxin S6b and S6c. Stimulation by endothelin was transmitted into cells as production of inositol 1,4,5-triphosphate and increase of calcium concentration in cytoplasm. In this experiment, the cells showed similar affinities for endothelin-1 and endothelin-3. In other words, the ratio of the affinity for endothelin-1 to that for endothelin-3 was in the range of 1:10 to 10:1. Therefore, it seems that this cDNA encodes one of the predicted subtypes of an endothelin receptor, which is non-specific to endothelin-1 and endothelin-3.

The above-determined DNA sequence encoding the endothelin receptor and the deduced amino acid sequence are set forth in Figs. 2A to 2C.

The DNA sequence and the amino acid sequence are described below.

There is an open reading frame of 1323b encoding 441 amino acid residues from the first ATG (initiation codon encoding methionine) to the stop codon of TGA (1324 - 1326). The 3' nontranslated region in the mRNA of the endothelin receptor encoded by this cDNA contains AUUUA sequence which destabilizes mRNA. This is analogous to transiently expressed cytokine or growth factor. The polypeptide starts from a signal sequence of 26 amino acid residues, which is predicted by von Heijne's algorithm (von Heijne, *Nucleic Acids Res.*, 14, 4683, 1989). The polypeptide consists of 415 amino acid residues, and the molecular weight of 46,901 is calculated.

The encoded polypeptide contains seven stretches of 24-28 hydrophobic amino acid residues, which are likely to represent transmembrane domains. The N-terminal region preceding the first putative transmembrane domain contains two potential N-glycosylation sites. These are common characteristics of receptors such as photoreceptor rhodopsin and other G protein-coupled receptors. The amino acid sequences which are likely to represent the transmembrane domains and the first and second extracellular domains are well preserved compared with the other G protein-coupled receptor superfamily. The third intracellular domain and the C-terminal region contains serine or threonine rich sites near lysine or arginine, which is similar to the other many G protein-coupled receptors. These sites may be phosphorylated by serine/threonine kinases. Other notable characteristics of the endothelin receptor include the relatively long

intracellular N-terminal portion (74 residues), the relatively short third intracellular portion (31 residues), the absence of an aspartate residue in the third transmembrane domain and the N-terminal signal sequence. These characteristics are partially analogous to those of the thyrotropin receptor, the LH-CG receptor and the tachykinin receptor.

5 The present invention provides the above-described DNA sequence encoding the mammalian endothelin receptor. The endothelin receptor DNA is preferably provided in a form which is capable of being expressed in a recombinant transcriptional unit under the control of mammalian, microbial, viral transcriptional or translational control elements. For example, a sequence to be expressed in a microorganism will contain no introns. In a preferred embodiment, the DNA sequence comprises at least one, but optionally  
10 more than one sequence component derived from a cDNA sequence or copy thereof.

The DNA sequences may be linked or flanked by DNA sequence prepared by assembly of synthetic oligonucleotides. However, synthetic genes assembled exclusively from oligonucleotides could be constructed using the sequence information provided herein. A representative sequence contains those substantially (essentially) identical to the nucleotide sequences shown in Figs. 2A to 2C. The coding  
15 sequences may include codons encoding one or more additional amino acids located at the N-terminus, for example, an N-terminal ATG codons specifying methionine linked with reading frame in the nucleotide sequence. Due to code degeneracy, there can be considerable variation in nucleotide sequences encoding the same amino acid sequence. Other embodiments include sequences capable of hybridizing to the representative sequence under moderately stringent conditions (42 °C, 20 % (v/v) formamide). The other  
20 sequences degenerate to those described above which encode biologically active endothelin receptor polypeptides.

The present invention also provides expression vectors for producing useful quantities of purified endothelin receptor. The vectors can comprise synthetic or cDNA derived DNA fragments encoding mammalian endothelin receptors or bioequivalent homologues operably linked to regulatory elements  
25 derived from mammalian, bacterial, yeast, bacteriophage or viral genes. Useful regulatory elements are described in greater detail below. Following transformation, transfection or infection of appropriate cell lines, such vectors can be induced to express recombinant protein.

Mammalian endothelin receptors can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems could also be employed to  
30 produce mammalian endothelin receptor using mRNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described by Pouwels et al. (*Cloning Vectors: A Laboratory Manual*, Elsevier, New York, 1985), the relevant disclosure of which is hereby incorporated by reference.

Various mammalian cell culture systems can be employed to express recombinant protein. Examples of  
35 suitable mammalian host cell lines include the COS-7 lines of monkey kidney cells, described by Gluzman (*Cell* 23:175, 1981), and other cell lines capable of expressing an appropriate vector, for example, C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors may comprise nontranscribed elements such as an origin of replication, a suitable promoter and enhancer, and other 5' or 3' flanking nontranscribed sequences, and 5' or 3' nontranslated sequences, such as necessary ribosome binding sites, a  
40 polyadenylation site, splice donor and acceptor sites, and termination sequences. DNA sequences derived from the SV40 viral genome, for example, SV40 replication origin, early promoter, enhancer, splice, and polyadenylation sites, may be used to provide the other genetic elements required for expression of a heterologous DNA sequence. Additional details regarding the use of a mammalian high expression vector to produce a recombinant mammalian endothelin receptor are provided in Examples 4 and 5, below.  
45 Exemplary vectors can be constructed as disclosed by Okayama and Berg (*Mol Cell Biol.* 3, 280, 1983).

A useful system for stable high level expression of mammalian receptor cDNAs in C127 rat mammary epithelial cells can be constructed substantially as described by Cosman et al. (*Molecular Immunol.* 23:935, 1986).

Yeast systems, preferably employing *Saccharomyces* species such as *S. cerevisiae*, can also be  
50 employed for expression of the recombinant proteins of the present invention. Yeast of other genera, for example, *Pichia* or *Kluyveromyces*, have also been employed as production strains for recombinant proteins.

Generally, useful yeast vectors will include origins of replication and selectable markers permitting transformation of both yeast and *E. coli*, e.g., the ampicillin resistance gene (Amp<sup>r</sup>) of *E. coli* and *S. cerevisiae* TRP1 gene, and a promoter derived from a highly-expressed yeast gene to induce transcription  
55 of a downstream structural gene. Such promoters can be derived from yeast transcriptional units encoding highly expressed genes such as 3-phosphoglycerate kinase (PGK),  $\alpha$ -factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate reading

frame with translation initiation and termination sequences, and, preferably, a leader sequence capable of directing secretion of translated protein into the extracellular medium. Optionally, the heterologous sequences can encode a fusion protein including an N-terminal identification peptide or other sequence imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

Useful yeast vectors can be assembled using DNA sequences from pBR322 (Amp<sup>r</sup> gene and origin of replication) for selection and replication in *E. coli* and yeast DNA sequences including a glucose-repressible alcohol dehydrogenase 2 (ADH2) promoter. The ADH2 promoter has been described by Russell et al. (*J. Biol. Chem.* 258:2674, 1982) and Beier et al. (*Nature* 300:724, 1982). Such vectors may also include a yeast TRP1 gene as a selectable marker and the yeast 2  $\mu$  origin of replication. A yeast leader sequence, for example, the  $\alpha$ -factor leader which directs secretion of heterologous proteins from a yeast host, can be inserted between the promoter and the structural gene to be expressed (see Kurian et al., U.S. Patent No. 4,546,082; Kurian et al., *Cell* 30:933, 1982); and Bittner et al., *Proc. Natl. Acad. Sci. USA* 81:983 1984).

The leader sequence may be modified to contain, near its 3' end, one or more useful restriction sites to facilitate fusion of the leader sequence to foreign genes.

Suitable yeast transformation protocols are known to those skilled in the art; an exemplary technique is described by Hinnen et al. (*Proc. Natl. Acad. Sci. USA* 75:1929, 1978), selecting for Trp<sup>+</sup> transformants in a selective medium consisting of 0.67 % yeast nitrogen source, 0.5 % casamino acids, 2 % glucose, 10  $\mu$ g/ml adenine and 20  $\mu$ g/ml uracil.

Host strains transformed by vectors comprising the ADH2 promoter may be grown for expression in a rich medium consisting of 1 % yeast extract, 2 % peptone and 1 % glucose supplemented with 80  $\mu$ g/ml adenine and 80  $\mu$ g/ml uracil. Dereglulation of the ADH2 promoter occurs upon exhaustion of medium glucose. Crude yeast supernatants are harvested by filtration and held at 4 °C prior to further purification.

Useful expression vectors for bacterial use are constructed by inserting a DNA sequence encoding mammalian endothelin receptor together with suitable translation initiation and termination signals in operable reading frame with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure growth within the host. Suitable prokaryotic hosts for transformation include *E. coli*, *Bacillus Subtilis*, *Salmonella typhimurium* and various species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, although others may also be employed as a matter of choice.

Expression vectors are conveniently constructed by cleavage of cDNA clones at sites close to the codon encoding the N-terminal residue of the mature protein. Synthetic oligonucleotides can then be used to "add back" any deleted sections of the coding region and to provide a linking sequence for ligation of the coding fragment in appropriate reading frame in the expression vector, and optionally a codon specifying an initiator methionine.

As a representative but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example pKK223-3p (Pharmacia Fine Chemicals, Uppsala, Sweden) and pGEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "main chain" sections are combined with an appropriate promoter and the structural sequence to be expressed.

A particularly useful bacterial expression system employs the phage  $\lambda$  P<sub>L</sub> promoter and cI857 thermolabile repressor. Plasmid vectors available from the American Type Culture Collection which incorporate derivatives of the  $\lambda$  P<sub>L</sub> promoter include plasmid pHUB2, resident in *E. coli* strain JMB9 (ATCC37092) and pPLc28, resident in *E. coli* RR1 (ATCC53082). Other useful promoters for expression in *E. coli* include the T7 RNA polymerase promoter described by Studier et al. (*J. Mol. Biol.* 189:113, 1986), the *lacZ* promoter described by Laner (*J. Mol. Appl. Genet.* 1:139-147, 1981) which is available as ATCC 37121, and the *tac* promoter described by Maniatis (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1982, p412) which is available as ATCC37138.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is derepressed by appropriate means (e.g., temperature shift or chemical induction) and cells cultured for an additional period. Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification. Cells are grown, for example, in a 10 liter fermenter employing conditions of maximum aeration and vigorous agitation. An antifoaming agent (Antifoam A) is preferably employed. Cultures are grown at 30 °C in the superinduction medium disclosed by Mott et al. (*Proc. Natl. Acad. Sci. USA* 82:88, 1985), alternatively including antibiotics, derepressed at a cell density corresponding to A<sub>600</sub> = 0.4-0.5 by elevating the temperature to 42 °C, and harvested for 2-20 hours, preferably 3-6 hours after the upward

temperature shift. The cell mass is initially concentrated by filtration or other means, then centrifuged at  $10,000 \times g$  (10,000 G) for 10 minutes at  $4^\circ\text{C}$ , followed by rapidly freezing the cell pellet.

Preferably, purified mammalian endothelin receptors or bioequivalent analogs are prepared by culturing suitable host/vector systems to express the recombinant translation products of the synthetic genes of the present invention, which are then purified from culture media.

An alternative process for producing purified endothelin receptor involves purification from cell culture supernatants or extracts. In this approach, a cell line which elaborates useful quantities of the protein is employed. Supernatants from such cell lines can be optionally concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Falcon ultra-filtration unit. Following the concentration step, the concentrate can be applied to a suitable purification matrix as previously described. For example, a suitable affinity matrix can comprise an endothelin receptor or lectin or antibody molecule bound to a suitable support. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups.

Finally, one or more reversed-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, e.g., silica gel having methyl or other aliphatic groups, can be employed to further purify an endothelin receptor composition. Some or all of the foregoing purification steps, in various combinations, can also be employed to provide a homogeneous recombinant protein.

Recombinant protein produced in bacterial culture is usually isolated by initial extraction from cell pellets, followed by one or more concentration, salting-out, aqueous ion exchange or gel filtration chromatography steps. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps. Microbial cells employed in expression of recombinant mammalian endothelin receptor can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption or use of cell lysing agents.

Fermentation of yeast which expresses mammalian endothelin receptor as a secreted protein greatly simplifies purification. Secreted recombinant protein resulting from a large-scale fermentation can be purified by methods analogous to those disclosed by Urdal et al. (*J. Chromatog.* 296:171, 1984). This reference describes two sequential, reversed-phase HPLC steps for purification of recombinant human GM-CSF on a preparative HPLC column.

In its various embodiments, the present invention provides substantially (essentially) homogeneous recombinant mammalian endothelin receptor polypeptides free of contaminating endogenous material.

Recombinant endothelin receptor proteins of the present invention also include suitable peptide or protein sequences employed as aids to expression in microorganisms or purification of microbially expressed proteins.

Bioequivalent analogues of the proteins of this invention include various analogs, for example, truncated versions of endothelin receptors wherein terminal residues or sequences, which exists in internal cell and are not needed for biological activity, are deleted.

Examples of the present invention are described below.

#### EXAMPLE 1

A cDNA library was constructed by a reverse transcription of poly(A)<sup>+</sup> RNA according to a procedure similar to that of Chirgwin et al. (*Biochem.*, 18, 5294, 1979). The poly(A)<sup>+</sup> RNA was isolated from total RNA extracted from rat lung. In more detail, the tissue was dissolved in a solution of guanidinium isothiocyanate. The solution was layered over a pad of CsCl, and was centrifuged to precipitate RNA. The RNA pellet was resuspended and further purified by protease digestion, organic extraction and alcohol precipitation. Poly(A)<sup>+</sup> RNA was isolated by oligo dT cellulose chromatography and double-stranded cDNA was prepared by a method similar to that of Gubler and Hoffman (*Gene* 25, 263, 1983). Briefly, the RNA was copied into cDNA by reverse transcriptase using either oligo dT or random oligonucleotides as primer. The cDNA was made double-stranded by incubation with *E. coli* DNA polymerase I and RNase H, and the ends made flush by further incubation with T<sub>4</sub> DNA polymerase. BstXI linker was added to the blunt-ended cDNA, and then short chains were removed by a gel filtration chromatography using Sepharose CL-2B. The cDNA was combined with a high expression plasmid vector for mammalian cells (pCDM8). A schematic illustration of pCDM8 is shown in Fig. 3.

The pCDM8 vector is a plasmid vector of 4.8 kb which contains a replication initiating point of SV 40 and polyoma and another replication initiating point of cytomegalo-virus/T7RNA polymerase promoter and

M13 (see Seed, *Nature*, 329, 840, 1987).

The resulting rat lung cDNA library on pCDM8 was used to transform *E. coli* (Mc 1061/P<sub>3</sub>) to provide about  $5 \times 10^5$  colonies. These recombinants were preserved in pools for each 500 colonies. The pooled DNA was used to transfect a sub-confluent layer of monkey COS-7 cells using DEAE-dextran followed by chloroquine treatment, as described by Seed et al., *Proc. Natl. Acad. Sci. USA*, 84, 3365, 1987). The cells were then grown in culture for three days to permit transient expression of the inserted sequences. The cell monolayers in each plate were assayed for endothelin receptor binding as follows. To each plate was added 2 ml of DMEM medium of 3 % BSA containing  $2.5 \times 10^{-11}$  M [<sup>125</sup>I-Tyr<sup>13</sup>]endothelin-1, and the plates were incubated for 2 hours at 37 °C at 5 % CO<sub>2</sub>. This medium was then discarded, and each plate was once washed with the above medium (containing no [<sup>125</sup>I-Tyr<sup>13</sup>]endothelin-1), and twice washed with PBS (pH 7.4). Each plate was then fixed with PBS containing 2.5 % glutaraldehyde. Each plate was dried in the air. The edges of each plate were broken off, leaving a flat disk which was contacted with a X-ray film for 8 to 72 hours at -80 °C. The endothelin-1 binding activity was visualized on the exposed films as a black spot. About  $2 \times 10^4$  recombinants were screened from the library as is mentioned above. Thus COS-7 recombinants transfected with a single clone which is capable of inducing expression of endothelin receptor were obtained.

The inserted fragments of clone prETR-7 were subcloned to pUC118/119 plasmid, and its DNA sequence was determined according to the dideoxy method (see Sanger et al., *Proc. Natl. Acad. Sci. USA*, 74, 5463, 1977).

## EXAMPLE 2

Characteristics of rat endothelin receptor expressed on COS-7 cells (information transport system from receptor)

### i) Production of inositol phosphates

The COS-7 cells transfected with prETR-7 were cultivated for 3 days, and were detached using 0.025 % trypsin/0.05 % EGTA. The reaction was stopped by soybean trypsin inhibitor, and the cells were collected by centrifugation. The cells were washed twice with a solution A (140 mM sodium chloride, 4 mM potassium chloride, 1 mM disodium phosphate, 1 mM magnesium chloride, 1.25 calcium chloride, 11 mM glucose, 5 mM HEPES (pH 7.4), 0.2 % BSA), and incubated for 3 hours in the solution A further containing 80 µCi/ml <sup>3</sup>H-myo-inositol. The cells were then stimulated with  $10^{-7}$  M endothelin-1 or endothelin-3 for 30 minutes in the presence of 10 mM lithium chloride. The inositol phosphates were separated by AG-1X8 anion exchange chromatography, and were quantified by measuring radio activity.

The results are set forth in Fig. 4.

### ii) Increase of calcium concentration in cytoplasm

The collected and washed COS-7 cells were incubated at 20 °C for 60 minutes in the solution A further containing 4 µM fura-2/AM. The cells were twice washed and preserved at 20 °C in the solution A (containing no fura-2/AM). For one experiment, about  $10^6$  cells in 1 ml were used. The cells were continuously stirred in cubet, and measured at excitation spectrum of 340 nm or 380 nm and at fluorescence spectrum of 500 nm. Endothelin-1, 2 or 3 of various concentration was injected by a microsyringe through a rubber septum. The calcium concentration was calculated from the ratio of the fluorescence strength according to the method of Grynkiewicz et al. (*J. Biol. Chem.*, 260, 3440, 1985).

EC<sub>50</sub> of the endothelin receptor expressed on COS-7 cells were about  $2 \times 10^{-11}$  M. The effects were analogous with respect to endothelin-1, 2 and 3.

The results are set forth in Fig. 5.

### iii) Binding experiment of [<sup>125</sup>I-Tyr<sup>13</sup>]endothelin-1

The COS-7 cells transfected with prETR-7 were cultivated in 12-holes plate, and washed once with the solution A. The cells were incubated at 37 °C for 60 minutes in 1 ml solution A containing [<sup>125</sup>I-Tyr<sup>13</sup>]endothelin-1 and endothelin-1, 2 or 3 of various concentration. The cells were well washed, and the radio activities binding the cell were measured.

The binding affinity of the endothelin receptor expressed on COS-7 cells was about  $2 \times 10^{-9}$  M. The affinities were analogous with respect to endothelin-1, 2 and 3.



The results are set forth in Fig. 6.

### EXAMPLE 3

#### Expression in tissues by northern blots using endothelin receptor cDNA

Poly(A)<sup>+</sup> RNA was extracted from various rat tissues in the same manner as is mentioned above. Each 10 µg of the RNAs was separated using formaldehyde/1.1 % agarose gel electrophoresis, and transferred to gene screen plus membrane (NEN, Dupont). Then, 2kb of cDNA fragments was labeled by α-<sup>32</sup>P-dCTP according to a random prim method to 8 × 10<sup>8</sup> c.p.m./mg, and used as a probe. Hybridization was carried out at 42 °C in a solution of 1M sodium chloride, 50 % formaldehyde, 1 % SDS and 250 µg salmon sperm DNA. The sample was washed with 2 × SSC/1 % SDS five times at 22 °C, once at 65 °C, and once washed with 0.1 × SSC/0.1 % SDS at 50 °C. Autoradiography was carried out for 10 hours.

Northern blots were carried out with respect to poly(A)<sup>+</sup> RNAs extracted from 14 rat tissues. As the results, 5.0 kb of endothelin receptor mRNA was detected in the tissues in which a physiological function of endothelin had been reported. A large amount of endothelin receptor mRNA was expressed in brain, lung, kidney, heart, eyeball liver, stomach and adrenal in order of amount. A relatively large amount was expressed in womb, small intestine and glandular submandibularis, a trace amount was expressed in testis and skeletal muscle, and there was no expression in spleen and aorta smooth muscle.

### EXAMPLE 4

#### Expression of endothelin receptor by dhfr-defective strain of CHO cell line

Endothelin receptor cDNA insert was integrated into the down stream of the promoter of an expression vector pSVD (see R.A. Poovman et al., *Proteins*, 1, 139, 1986) which contains dhfr (dihydroforate reductase) gene and a promoter derived from SV40 virus. Thus an endothelin receptor expression plasmid pSVDreTR was prepared.

The endothelin receptor expression plasmid was set forth in Fig. 7.

CHO (chinese hamster ovary) dhfr<sup>-</sup> cells (see G. Urlaub et al., *Proc. Natl. Acad. Sci. USA* 77, 4216, 1980) were cultivated as a subconfluent monolayer in a HamF12 medium containing 10 % FBS.

The CHO dhfr<sup>-</sup> cells were transfected with the endothelin receptor expression plasmid pSVDreTR according to the calcium phosphate method and glycerol treatment. After 24 hours, the transfected cells were subcultured in an area about 20 times as large as the previous area. After 24 hours, the cells were well inoculated, and the medium was replaced with DMEM medium containing 10 % dialysis FBS.

Thus only the cells transfected with dhfr gene into genome grew and their colony was formed.

The well grown colony was detached using trypsin according to the penicillin cup method, and subcultured in 12 holes plate using the same DMEM medium.

Thus isolated clonal cells expressed endothelin receptor, which was confirmed by the observation of increase of calcium concentration in cytoplasm using fura-2 when endothelin was added to the cells as described at ii) in Example 2. The expression was also confirmed by the binding experiment of [<sup>125</sup>I-Tyr<sup>13</sup>] endothelin-1 as described at iii) in Example 2.

### EXAMPLE 5

#### Expression of endothelin receptor by L cell

Fragments inserted with human endothelin receptor cDNA was integrated into the down stream of the promoter of an expression vector pME18Sf<sup>-</sup> which contains the SRa promoter.

L cells (mouse fibroblast) were cultivated as a subconfluent monolayer in a DMEM medium containing 10 % FBS.

The L cells were stably transfected with the human endothelin receptor expression plasmid and a plasmid pSVneo containing neomycin resistant gene according to the calcium phosphate method and glycerol treatment. After 24 hours, the transfected cells were subcultured in an area about 10 times as large as the previous area. After 24 hours, the cells were well inoculated, and the medium was replaced with DMEM medium containing 10 % FBS and 0.5 mg/ml of neomycin.

Thus only the cells transfected with neomycin resistant gene into genome grew and their colony was formed. The human endothelin receptor expression plasmid is usually integrated simultaneously with the

integration of the plasmid pSVneo.

The well grown colony was detached using trypsin according to the penicillin cup method, and subcultured in 12 holes plate using the same DMEM medium.

Thus isolated clonal cells expressed endothelin receptor, which was confirmed by the observation of  
5 increase of calcium concentration in cytoplasm using fura-2 when endothelin was added to the cells as described at ii) in Example 2.

The affinities of the cells transfected with the human endothelin receptor expression plasmid were analogous with respect to endothelin-1 and 3.

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## Specimen Sequence Listing

SEQ ID NO: 1

SEQUENCE TYPE: Protein

SEQUENCE LENGTH: 441 amino acids

ORIGINAL SOURCE

ORGANISM: rat

IMMEDIATE EXPERIMENTAL SOURCE

NAME OF CELL LINE: rat lung

PROPERTIES: endothelin receptor

Met	Gln	Ser	Ser	Ala	Ser	Arg	Cys	Gly	Arg	Ala	Leu	Val	Ala	Leu
1				5					10					15

Leu	Leu	Ala	Cys	Gly	Leu	Leu	Gly	Val	Trp	Gly	Glu	Lys	Arg	Gly
			20						25					30

Phe	Pro	Pro	Ala	Gln	Ala	Thr	Pro	Ser	Leu	Leu	Gly	Thr	Lys	Glu
			35						40					45

Val	Met	Thr	Pro	Pro	Thr	Lys	Thr	Ser	Trp	Thr	Arg	Gly	Ser	Asn
			50						55					60

Ser	Ser	Leu	Met	Arg	Phe	Arg	Thr	Ala	Glu	Val	Thr	Lys	Gly	Gly
			65						70					75

Arg	Val	Ala	Gly	Val	Pro	Pro	Arg	Ser	Phe	Pro	Pro	Pro	Cys	Gln
			80						85					90

Arg	Lys	Ile	Glu	Ile	Asn	Lys	Thr	Phe	Lys	Tyr	Ile	Asn	Thr	Ile
			95						100					105

Val	Ser	Cys	Leu	Val	Phe	Val	Leu	Gly	Ile	Ile	Gly	Asn	Ser	Thr
			110						115					120

Leu	Leu	Arg	Ile	Ile	Tyr	Lys	Asn	Lys	Cys	Met	Arg	Asn	Gly	Pro
			125						130					135

Asn	Ile	Leu	Ile	Ala	Ser	Leu	Ala	Leu	Gly	Asp	Leu	Leu	His	Ile
			140						145					150

Ile	Ile	Asp	Ile	Pro	Ile	Asn	Ala	Tyr	Lys	Leu	Leu	Ala	Gly	Asp
			155						160					165

Trp	Pro	Phe	Gly	Ala	Glu	Met	Cys	Lys	Leu	Val	Pro	Phe	Ile	Gln
			170						175					180

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	Lys	Ala	Ser	Val	Gly	Ile	Thr	Val	Leu	Ser	Leu	Cys	Ala	Leu	Ser	185	190	195
5	Ile	Asp	Arg	Tyr	Arg	Ala	Val	Ala	Ser	Trp	Ser	Arg	Ile	Lys	Gly	200	205	210
	Ile	Gly	Val	Pro	Lys	Trp	Thr	Ala	Val	Glu	Ile	Val	Leu	Ile	Trp	215	220	225
10	Val	Val	Ser	Val	Val	Leu	Ala	Val	Pro	Glu	Ala	Ile	Gly	Phe	Asp	230	235	240
	Val	Ile	Thr	Ser	Asp	Tyr	Lys	Gly	Lys	Pro	Leu	Arg	Val	Cys	Met	245	250	255
15	Leu	Asn	Pro	Phe	Gln	Lys	Thr	Ala	Phe	Met	Gln	Phe	Tyr	Lys	Thr	260	265	270
	Ala	Lys	Asp	Trp	Trp	Leu	Phe	Ser	Phe	Tyr	Phe	Cys	Leu	Pro	Leu	275	280	285
20	Ala	Ile	Thr	Ala	Ile	Phe	Tyr	Thr	Leu	Met	Thr	Cys	Glu	Met	Leu	290	295	300
	Arg	Lys	Lys	Ser	Gly	Met	Gln	Ile	Ala	Leu	Asn	Asp	His	Leu	Lys	305	310	315
25	Gln	Arg	Arg	Glu	Val	Ala	Lys	Thr	Val	Phe	Cys	Leu	Val	Leu	Val	320	325	330
	Phe	Ala	Leu	Cys	Trp	Leu	Pro	Leu	His	Leu	Ser	Arg	Ile	Leu	Lys	335	340	345
30	Leu	Thr	Leu	Tyr	Asp	Gln	Ser	Asn	Pro	Gln	Arg	Cys	Glu	Leu	Leu	350	355	360
	Ser	Phe	Leu	Leu	Val	Leu	Asp	Tyr	Ile	Gly	Ile	Asn	Met	Ala	Ser	365	370	375
35	Leu	Asn	Ser	Cys	Ile	Asn	Pro	Ile	Ala	Leu	Tyr	Leu	Val	Ser	Lys	380	385	390
	Arg	Phe	Lys	Asn	Cys	Phe	Lys	Ser	Cys	Leu	Cys	Cys	Trp	Cys	Gln	395	400	405
40	Thr	Phe	Glu	Glu	Lys	Gln	Ser	Leu	Glu	Glu	Lys	Gln	Ser	Cys	Leu	410	415	420
45	Lys	Phe	Lys	Ala	Asn	Asp	His	Gly	Tyr	Asp	Asn	Phe	Arg	Ser	Ser	425	430	435
	Asn	Lys	Tyr	Ser	Ser	Ser										440		
50																		
55																		

SEQ ID NO: 2

SEQUENCE TYPE: Nucleotide with corresponding protein

SEQUENCE LENGTH: 1965 base pairs

STRANDEDNESS: double

TOPOLOGY: linear

MOLECULE TYPE: cDNA

ORIGINAL SOURCE

ORGANISM: rat

IMMEDIATE EXPERIMENTAL SOURCE

NAME OF CELL LINE: rat lung

PROPERTIES: endothelin receptor

GGTGGCGTGC GCCCAAGTTC CCCATTGGCG CGCAAACCTTA ACTTACTGTT 50

GTGGCGCGGG TAGAGACAAC CCGGCTAGGG TGAGTGTTTT CAGAGGCGTG 100

GCTGGGTAGC TGAATAAGT ACCCTCTCTT CATTCCCCTG TTGTTCTCCA 150

GACTGAAAAC GGCGGAGCGG CTACGGGACT CTCACAGGAG CAAGCTGCAA 200

C ATG CAA TCG TCC GCA AGC CGG TGC GGA CGC GCC TTG 237

Met Gln Ser Ser Ala Ser Arg Cys Gly Arg Ala Leu  
1 5 10

GTG GCG CTG CTG CTG GCC TGT GGC TTG TTG GGG GTA 273

Val Ala Leu Leu Leu Ala Cys Gly Leu Leu Gly Val  
15 20

TGG GGA GAG AAA AGA GGA TTC CCA CCT GCC CAG GCC 309

Trp Gly Glu Lys Arg Gly Phe Pro Pro Ala Gln Ala  
25 30 35

ACA CCA TCT CTT CTC GGG ACT AAA GAA GTT ATG ACG 345

Thr Pro Ser Leu Leu Gly Thr Lys Glu Val Met Thr  
40 45

CCA CCC ACT AAG ACC TCC TGG ACT AGA GGT TCC AAC 381

Pro Pro Thr Lys Thr Ser Trp Thr Arg Gly Ser Asn  
50 55 60

TCC AGT CTG ATG CGT TTC CGC ACT GCG GAG GTG ACC 417

Ser Ser Leu Met Arg Phe Arg Thr Ala Glu Val Thr  
65 70

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5	AAA GGA GGG AGG GTG GCT GGA GTC CCG CCA AGA TCC Lys Gly Gly Arg Val Ala Gly Val Pro Pro Arg Ser 75 80	453
10	TTC CCT CCT CCG TGC CAA CGA AAA ATT GAG ATC AAC Phe Pro Pro Pro Cys Gln Arg Lys Ile Glu Ile Asn 85 90 95	489
15	AAG ACT TTT AAA TAC ATC AAC ACG ATT GTA TCA TGC Lys Thr Phe Lys Tyr Ile Asn Thr Ile Val Ser Cys 100 105	525
20	CTC GTG TTC GTG CTA GGC ATC ATC GGG AAC TCC ACA Leu Val Phe Val Leu Gly Ile Ile Gly Asn Ser Thr 110 115 120	561
25	CTG CTA AGA ATC ATC TAC AAG AAC AAG TGC ATG AGA Leu Leu Arg Ile Ile Tyr Lys Asn Lys Cys Met Arg 125 130	597
30	AAT GGT CCC AAT ATC TTG ATC GCC AGC CTG GCT CTG Asn Gly Pro Asn Ile Leu Ile Ala Ser Leu Ala Leu 135 140	633
35	GGA GAT CTG CTA CAC ATC ATC ATC GAC ATT CCC ATT Gly Asp Leu Leu His Ile Ile Ile Asp Ile Pro Ile 145 150 155	669
40	AAT GCC TAC AAG CTG CTG GCA GGG GAC TGG CCA TTT Asn Ala Tyr Lys Leu Leu Ala Gly Asp Trp Pro Phe 160 165	705
45	GGA GCT GAG ATG TGC AAG CTG GTG CCC TTC ATA CAG Gly Ala Glu Met Cys Lys Leu Val Pro Phe Ile Gln 170 175 180	741
50	AAG GCT TCT GTG GGG ATC ACA GTG TTG AGT CTA TGT Lys Ala Ser Val Gly Ile Thr Val Leu Ser Leu Cys 185 190	777
55	GCT CTA AGT ATT GAC AGA TAT CGA GCT GTT GCT TCT Ala Leu Ser Ile Asp Arg Tyr Arg Ala Val Ala Ser 195 200	813
	TGG AGT CGA ATT AAA GGA ATT GGG GTT CCA AAA TGG Trp Ser Arg Ile Lys Gly Ile Gly Val Pro Lys Trp 205 210 215	849
	ACA GCA GTA GAA ATT GTT TTA ATT TGG GTG GTC TCT Thr Ala Val Glu Ile Val Leu Ile Trp Val Val Ser 220 225	885
	GTG GTT CTG GCT GTC CCT GAA GCC ATA GGT TTT GAT Val Val Leu Ala Val Pro Glu Ala Ile Gly Phe Asp 230 235 240	921

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	GTG	ATT	ACG	TCG	GAC	TAC	AAA	GGA	AAG	CCC	CTA	AGG	957
	Val	Ile	Thr	Ser	Asp	Tyr	Lys	Gly	Lys	Pro	Leu	Arg	
					245					250			
5	GTC	TGC	ATG	CTT	AAT	CCC	TTT	CAG	AAA	ACA	GCC	TTC	993
	Val	Cys	Met	Leu	Asn	Pro	Phe	Gln	Lys	Thr	Ala	Phe	
			255					260					
10	ATG	CAG	TTT	TAC	AAG	ACA	GCC	AAA	GAC	TGG	TGG	CTG	1029
	Met	Gln	Phe	Tyr	Lys	Thr	Ala	Lys	Asp	Trp	Trp	Leu	
	265					270					275		
15	TTC	AGT	TTC	TAC	TTC	TGC	TTG	CCG	CTA	GCC	ATC	ACT	1065
	Phe	Ser	Phe	Tyr	Phe	Cys	Leu	Pro	Leu	Ala	Ile	Thr	
				280					285				
20	GCG	ATC	TTT	TAC	ACC	CTA	ATG	ACC	TGT	GAG	ATG	CTC	1101
	Ala	Ile	Phe	Tyr	Thr	Leu	Met	Thr	Cys	Glu	Met	Leu	
			290				295					300	
25	AGA	AAG	AAA	AGT	GGT	ATG	CAG	ATT	GCC	TTG	AAT	GAC	1137
	Arg	Lys	Lys	Ser	Gly	Met	Gln	Ile	Ala	Leu	Asn	Asp	
				305						310			
30	CAC	TTA	AAG	CAG	AGA	CGA	GAA	GTG	GCC	AAG	ACA	GTA	1173
	His	Leu	Lys	Gln	Arg	Arg	Glu	Val	Ala	Lys	Thr	Val	
			315					320					
35	TTC	TGC	CTG	GTC	CTC	GTG	TTT	GCC	CTC	TGT	TGG	CTT	1209
	Phe	Cys	Leu	Val	Leu	Val	Phe	Ala	Leu	Cys	Trp	Leu	
	325					330					335		
40	CCC	CTT	CAC	CTC	AGC	AGG	ATT	CTG	AAG	CTC	ACC	CTT	1245
	Pro	Leu	His	Leu	Ser	Arg	Ile	Leu	Lys	Leu	Thr	Leu	
				340					345				
45	TAT	GAC	CAG	AGC	AAT	CCT	CAG	AGG	TGT	GAA	CTT	CTG	1281
	Tyr	Asp	Gln	Ser	Asn	Pro	Gln	Arg	Cys	Glu	Leu	Leu	
		350					355					360	
50	AGT	TTT	TTG	CTG	GTT	TTG	GAC	TAC	ATT	GGT	ATC	AAC	1317
	Ser	Phe	Leu	Leu	Val	Leu	Asp	Tyr	Ile	Gly	Ile	Asn	
					365					370			
55	ATG	GCT	TCT	TTG	AAT	TCC	TGC	ATT	AAT	CCA	ATC	GCT	1353
	Met	Ala	Ser	Leu	Asn	Ser	Cys	Ile	Asn	Pro	Ile	Ala	
			375					380					
60	CTG	TAT	TTG	GTG	AGC	AAG	AGA	TTC	AAA	AAC	TGC	TTT	1389
	Leu	Tyr	Leu	Val	Ser	Lys	Arg	Phe	Lys	Asn	Cys	Phe	
	385					390					395		

AAG TCG TGT TTG TGC TGC TGG TGC CAA ACG TTT GAG 1425  
 Lys Ser Cys Leu Cys Cys Trp Cys Gln Thr Phe Glu  
 400 405

5 GAA AAA CAG TCC TTA GAG GAG AAG CAA TCC TGC TTG 1461  
 Glu Lys Gln Ser Leu Glu Glu Lys Gln Ser Cys Leu  
 410 415 420

10 AAG TTC AAA GCT AAC GAT CAC GGA TAC GAC AAC TTC 1497  
 Lys Phe Lys Ala Asn Asp His Gly Tyr Asp Asn Phe  
 425 430

CGC TCC AGC AAT AAA TAC AGC TCA TCT 1524  
 Arg Ser Ser Asn Lys Tyr Ser Ser Ser  
 435 440

15 TGAAGGAAGG AACACTCACT GAATCTCATT GTCCTCATCG TGGACAGATA 1574  
 GCATTAAAAC AAAATGAAAC CTTTGCCAAA CCCAAACGGA AAACCGTGCT 1624  
 20 TGC GGAAAGG TGTGCACGCA TGGGAGAGGG ATTGTTTTTT AACCGTTCTA 1674  
 ACTTTCCACA CCTGATATTT CACGGGCTGT TTACAACCTA AGAAAGCCAT 1724  
 GGAATGAAT GAAGCCTCGG GAAAGCACTT AGATTCTTAG TCAAGCACTT 1774  
 25 CAGCACGGCT CTAAAAGCC CTCCTGCAC TCACAGCCCA CTTACATTTA 1824  
 AAAACAAGAA CTCAACTCT ATTCAGGGGT TTATTATCCA GTCCTATGAA 1874  
 TCTGGATACA GGAATGCATG ACATTGCAAA ACAATTCTTA AAGCAAAGTT 1924  
 30 TCAATTGCTC GATTGAGAC AAAAAACAA AAAAAAAAAA A 1965

### 35 Claims

1. A DNA sequence substantially encoding a mammalian endothelin receptor.
2. The DNA sequence as claimed in claim 1, wherein the mammalian endothelin receptor has such a pharmacological activity that the ratio of the affinity of the receptor for endothelin-1 to that for endothelin-3 is in the range of 1:10 to 10:1.
3. The DNA sequence as claimed in claim 1, wherein the sequence comprises cDNA clones having a nucleotide sequence derived from the coding region of a native mammalian endothelin receptor gene.
4. The DNA sequence as claimed in claim 1, wherein the sequence is capable of hybridization to cDNA clones under moderately stringent conditions and encodes a biologically active endothelin receptor protein, said cDNA clones having a nucleotide sequence derived from the coding region of a native mammalian endothelin receptor gene.
5. The DNA sequence as claimed in claim 1, wherein the sequence is degenerate as a result of the genetic code to cDNA clones and encodes a biologically active endothelin receptor protein, said cDNA clones having a nucleotide sequence derived from the coding region of a native mammalian endothelin receptor gene.
6. The DNA sequence as claimed in claim 1, wherein the sequence is degenerate as a result of the genetic code to a sequence capable of hybridization to cDNA clones under moderately stringent conditions and encodes a biologically active endothelin receptor protein, said cDNA clones having a



nucleotide sequence derived from the coding region of a native mammalian endothelin receptor gene.

7. The DNA sequence as claimed in claim 1, wherein the sequence consists essentially of a synthetic gene which encodes a mammalian endothelin receptor protein which is capable of being expressed in a recombinant transcriptional unit comprising inducible regulatory elements derived from a microbial or viral operon.
8. The DNA sequence as claimed in claim 1, wherein the sequence has at least 30 % similarity to a DNA sequence encoding a mammalian endothelin receptor.
9. The DNA sequence as claimed in claim 1, wherein the sequence has at least 50 % similarity to a DNA sequence encoding a mammalian endothelin receptor.
10. The DNA sequence as claimed in claim 1, wherein the sequence has at least 80 % similarity to a DNA sequence encoding a mammalian endothelin receptor.
11. A recombinant expression vector which contains a DNA sequence substantially encoding a mammalian endothelin receptor.
12. A process for production of a mammalian endothelin receptor or an analogue thereof, which comprises transfecting a host cell with a recombinant expression vector and culturing the cell under conditions promoting expression, said vector containing a DNA sequence substantially encoding a mammalian endothelin receptor.
13. A protein composition containing a biologically active mammalian endothelin receptor or an analogue thereof which is produced by a recombinant cell culture.
14. An agent for detecting a mammalian endothelin, wherein the agent contains a biologically active mammalian endothelin receptor or an analogue thereof.

## FIG. 1A

Met-Gln-Ser-Ser-Ala-Ser-Arg-Cys-Gly-Arg-Ala-  
Leu-Val-Ala-Leu-Leu-Leu-Ala-Cys-Gly-Leu-Leu-  
Gly-Val-Trp-Gly-Glu-Lys-Arg-Gly-Phe-Pro-Pro-  
Ala-Gln-Ala-Thr-Pro-Ser-Leu-Leu-Gly-Thr-Lys-  
Glu-Val-Met-Thr-Pro-Pro-Thr-Lys-Thr-Ser-Trp-  
Thr-Arg-Gly-Ser-Asn-Ser-Ser-Leu-Met-Arg-Phe-  
Arg-Thr-Ala-Glu-Val-Thr-Lys-Gly-Gly-Arg-Val-  
Ala-Gly-Val-Pro-Pro-Arg-Ser-Phe-Pro-Pro-Pro-  
Cys-Gln-Arg-Lys-Ile-Glu-Ile-Asn-Lys-Thr-Phe-  
Lys-Tyr-Ile-Asn-Thr-Ile-Val-Ser-Cys-Leu-Val-  
Phe-Val-Leu-Gly-Ile-Ile-Gly-Asn-Ser-Thr-Leu-  
Leu-Arg-Ile-Ile-Tyr-Lys-Asn-Lys-Cys-Met-Arg-  
Asn-Gly-Pro-Asn-Ile-Leu-Ile-Ala-Ser-Leu-Ala-  
Leu-Gly-Asp-Leu-Leu-His-Ile-Ile-Ile-Asp-Ile-  
Pro-Ile-Asn-Ala-Tyr-Lys-Leu-Leu-Ala-Gly-Asp-  
Trp-Pro-Phe-Gly-Ala-Glu-Met-Cys-Lys-Leu-Val-  
Pro-Phe-Ile-Gln-Lys-Ala-Ser-Val-Gly-Ile-Thr-  
Val-Leu-Ser-Leu-Cys-Ala-Leu-Ser-Ile-Asp-Arg-  
Tyr-Arg-Ala-Val-Ala-Ser-Trp-Ser-Arg-Ile-Lys-  
Gly-Ile-Gly-Val-Pro-Lys-Trp-Thr-Ala-Val-Glu-  
Ile-Val-Leu-Ile-Trp-Val-Val-Ser-Val-Val-Leu-  
Ala-Val-Pro-Glu-Ala-Ile-Gly-Phe-Asp-Val-Ile-

## FIG. 1B

Thr-Ser-Asp-Tyr-Lys-Gly-Lys-Pro-Leu-Arg-Val-  
Cys-Met-Leu-Asn-Pro-Phe-Gln-Lys-Thr-Ala-Phe-  
Met-Gln-Phe-Tyr-Lys-Thr-Ala-Lys-Asp-Trp-Trp-  
Leu-Phe-Ser-Phe-Tyr-Phe-Cys-Leu-Pro-Leu-Ala-  
Ile-Thr-Ala-Ile-Phe-Tyr-Thr-Leu-Met-Thr-Cys-  
Glu-Met-Leu-Arg-Lys-Lys-Ser-Gly-Met-Gln-Ile-  
Ala-Leu-Asn-Asp-His-Leu-Lys-Gln-Arg-Arg-Glu-  
Val-Ala-Lys-Thr-Val-Phe-Cys-Leu-Val-Leu-Val-  
Phe-Ala-Leu-Cys-Trp-Leu-Pro-Leu-His-Leu-Ser-  
Arg-Ile-Leu-Lys-Leu-Thr-Leu-Tyr-Asp-Gln-Ser-  
Asn-Pro-Gln-Arg-Cys-Glu-Leu-Leu-Ser-Phe-Leu-  
Leu-Val-Leu-Asp-Tyr-Ile-Gly-Ile-Asn-Met-Ala-  
Ser-Leu-Asn-Ser-Cys-Ile-Asn-Pro-Ile-Ala-Leu-  
Tyr-Leu-Val-Ser-Lys-Arg-Phe-Lys-Asn-Cys-Phe-  
Lys-Ser-Cys-Leu-Cys-Cys-Trp-Cys-Gln-Thr-Phe-  
Glu-Glu-Lys-Gln-Ser-Leu-Glu-Glu-Lys-Gln-Ser-  
Cys-Leu-Lys-Phe-Lys-Ala-Asn-Asp-His-Gly-Tyr-  
Asp-Asn-Phe-Arg-Ser-Ser-Asn-Lys-Tyr-Ser-Ser-  
Ser

## FIG. 2A

-202	GGTGGCGTGCGCCCAAGTTCCTCCATTGGCGCGCAAACTTAACTTA	-158
-157	CTGTTGTGGCGCGGGTAGAGACAACCCGGCTAGGGTGAGTGTTTT	-113
-112	CAGAGGCGTGGCTGGGTAGCTGACTAAAGTACCCTCTCTTCATTC	-68
-67	CCCTGTTGTTCTCCAGACTGAAAACGGCGGAGCGGCTACGGGACT	-23
-22	CTCACAGGAGCAAGCTGCAACATGCAATCGTCCGCAAGCCGGTGC	22
	MetGlnSerSerAlaSerArgCys	
23	GGACGCGCCTTGGTGGCGCTGCTGCTGGCCTGTGGCTTGTGGGG	67
	GlyArgAlaLeuValAlaLeuLeuLeuAlaCysGlyLeuLeuGly	
68	GTATGGGGAGAGAAAAGAGGATTCCACCTGCCAGGCCACACCA	112
	ValTrpGlyGluLysArgGlyPheProProAlaGlnAlaThrPro	
113	TCTCTTCTCGGGACTAAAGAAGTTATGACGCCACCCACTAAGACC	157
	SerLeuLeuGlyThrLysGluValMetThrProProThrLysThr	
158	TCCTGGACTAGAGGTTCCAACCTCCAGTCTGATGCGTTTCCGCACT	202
	SerTrpThrArgGlySerAsnSerSerLeuMetArgPheArgThr	
203	GCGGAGGTGACCAAAGGAGGGAGGGTGGCTGGAGTCCCGCCAAGA	247
	AlaGluValThrLysGlyGlyArgValAlaGlyValProProArg	
248	TCCTTCCCTCCTCCGTGCCAACGAAAAATTGAGATCAACAAGACT	292
	SerPheProProProCysGlnArgLysIleGluIleAsnLysThr	
293	TTTAAATACATCAACACGATTGTATCATGCCTCGTGTTTCGTGCTA	337
	PheLysTyrIleAsnThrIleValSerCysLeuValPheValLeu	
338	GGCATCATCGGGAACCTCCACACTGCTAAGAATCATCTACAAGAAC	382
	GlyIleIleGlyAsnSerThrLeuLeuArgIleIleTyrLysAsn	
383	AAGTGCATGAGAAATGGTCCCAATATCTTGATCGCCAGCCTGGCT	427
	LysCysMetArgAsnGlyProAsnIleLeuIleAlaSerLeuAla	
428	CTGGGAGATCTGCTACACATCATCATCGACATTCCCATTAATGCC	472
	LeuGlyAspLeuLeuHisIleIleIleAspIleProIleAsnAla	
473	TACAAGCTGCTGGCAGGGGACTGGCCATTTGGAGCTGAGATGTGC	517
	TyrLysLeuLeuAlaGlyAspTrpProPheGlyAlaGluMetCys	
518	AAGCTGGTGGCCTTCATACAGAAGGCTTCTGTGGGGATCACAGTG	562
	LysLeuValProPheIleGlnLysAlaSerValGlyIleThrVal	

## FIG. 2B

563	TTGAGTCTATGTGCTCTAAGTATTGACAGATATCGAGCTGTTGCT	607
	LeuSerLeuCysAlaLeuSerIleAspArgTyrArgAlaValAla	
608	TCTTGGAGTCGAATTAAAGGAATTGGGGTTCCAAAATGGACAGCA	652
	SerTrpSerArgIleLysGlyIleGlyValProLysTrpThrAla	
653	GTAGAAATTGTTTTAATTGGGTGGTCTCTGTGGTTCTGGCTGTC	697
	ValGluIleValLeuIleTrpValValSerValValLeuAlaVal	
698	CCTGAAGCCATAGGTTTTGATGTGATTACGTCGGACTACAAAGGA	742
	ProGluAlaIleGlyPheAspValIleThrSerAspTyrLysGly	
743	AAGCCCCTAAGGGTCTGCATGCTTAATCCCTTTCAGAAACAGCC	787
	LysProLeuArgValCysMetLeuAsnProPheGlnLysThrAla	
788	TTCATGCAGTTTTACAAGACAGCCAAAGACTGGTGGCTGTTTCAGT	832
	PheMetGlnPheTyrLysThrAlaLysAspTrpTrpLeuPheSer	
833	TTCTACTTCTGCTTGCCGCTAGCCATCACTGCGATCTTTTACACC	877
	PheTyrPheCysLeuProLeuAlaIleThrAlaIlePheTyrThr	
878	CTAATGACCTGTGAGATGCTCAGAAAGAAAAGTGGTATGCAGATT	922
	LeuMetThrCysGluMetLeuArgLysLysSerGlyMetGlnIle	
923	GCCTTGAATGACCACTTAAAGCAGAGACGAGAAGTGGCCAAGACA	967
	AlaLeuAsnAspHisLeuLysGlnArgArgGluValAlaLysThr	
968	GTATTCTGCCTGGTCCTCGTGTGTTGCCCTCTGTTGGCTTCCCCTT	1012
	ValPheCysLeuValLeuValPheAlaLeuCysTrpLeuProLeu	
1013	CACCTCAGCAGGATTCTGAAGCTCACCTTTATGACCAGAGCAAT	1057
	HisLeuSerArgIleLeuLysLeuThrLeuTyrAspGlnSerAsn	
1058	CCTCAGAGGTGTGAACCTTCTGAGTTTTTGGTGGTTTTGGACTAC	1102
	ProGlnArgCysGluLeuLeuSerPheLeuLeuValLeuAspTyr	
1103	ATTGGTATCAACATGGCTTCTTTGAATTCCTGCATTAATCCAATC	1147
	IleGlyIleAsnMetAlaSerLeuAsnSerCysIleAsnProIle	
1148	GCTCTGTATTTGGTGAGCAAGAGATTCAAAAAGTCTTTAAGTCG	1192
	AlaLeuTyrLeuValSerLysArgPheLysAsnCysPheLysSer	
1193	TGTTTGTGCTGCTGGTGCCAAACGTTTGAGGAAAAACAGTCCTTA	1237
	CysLeuCysCysTrpCysGlnThrPheGluGluLysGlnSerLeu	
1238	GAGGAGAAGCAATCCTGCTTGAAGTTCAAAGCTAACGATCACGGA	1282
	GluGluLysGlnSerCysLeuLysPheLysAlaAsnAspHisGly	

## FIG. 2C

1283	TACGACAACTTCCGCTCCAGCAATAAATACAGCTCATCTTGAAGG	1327
	TyrAspAsnPheArgSerSerAsnLysTyrSerSerSer***	
1328	AAGGAACACTCACTGAATCTCATTGTCCTCATCGTGGACAGATAG	1372
1373	CATTAAAACAAAATGAAACCTTTGCCAAACCCAAACGGAAAACCG	1417
1418	TGCTTGCGGAAAGGTGTGCACGCATGGGAGAGGGATTGTTTTTTA	1462
1463	ACCGTTCTAACTTTCCACACCTGATATTTACGGGCTGTTTACAA	1507
1508	CCTAAGAAAGCCATGGGAATGAATGAAGCCTCGGGAAAGCACTTA	1552
1553	GATTCTTAGTCAAGCACTTCAGCACGGCTCTTAAAAGCCCTCACT	1597
1598	GCACTCACAGCCCACCTTACATTTAAAAACAAGAACTCAAACCTCTA	1642
1643	TTCAGGGGTTTATTATCCAGTCCTATGAATCTGGATACAGGAATG	1687
1688	CATGACATTGCAAAACAATTCTTAAAGCAAAGTTTCAATTGCTCG	1732
1733	ATTTGAGACAAAAACAAAACAAAAA	1762

FIG. 3

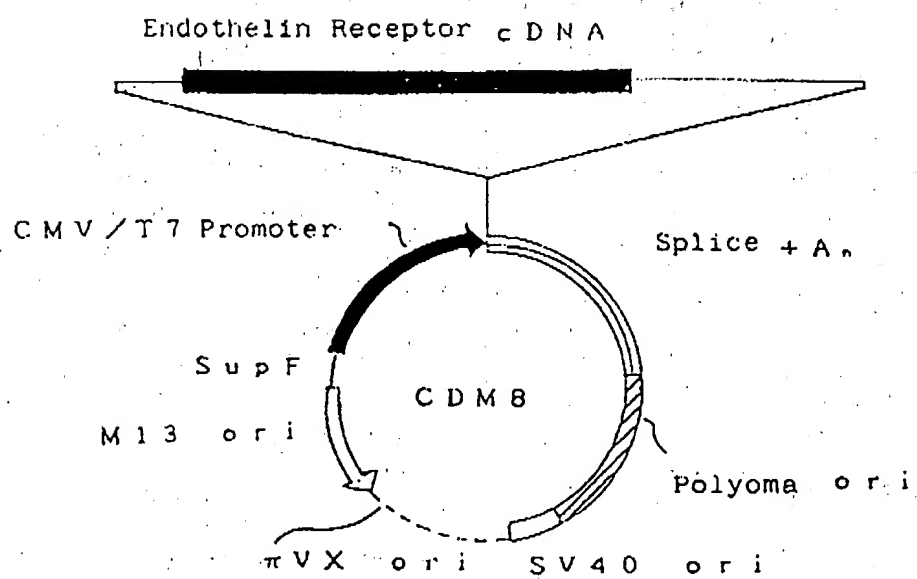


FIG. 4

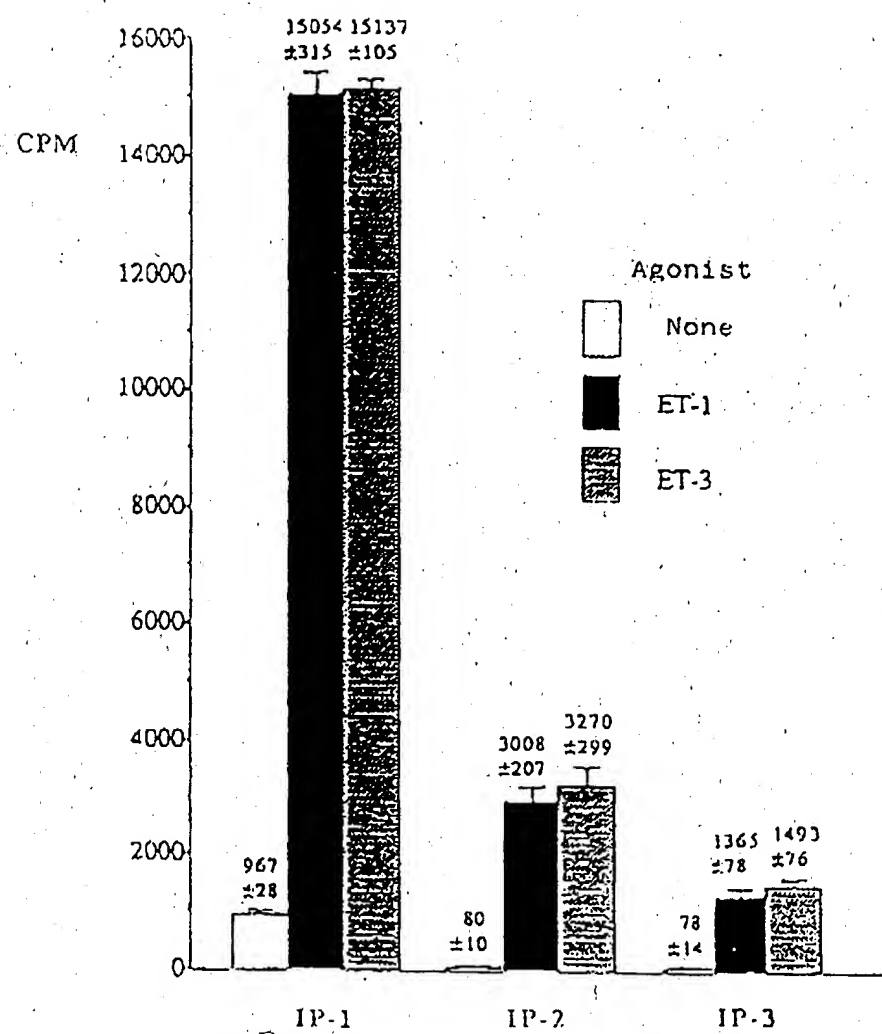




FIG. 5

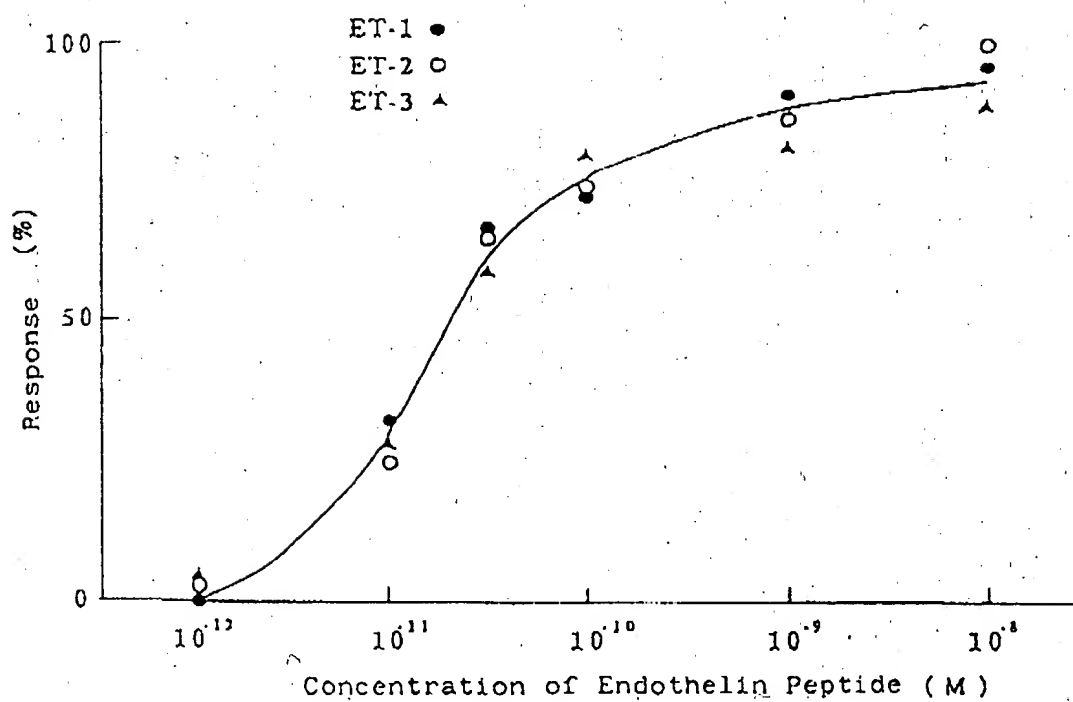


FIG. 6

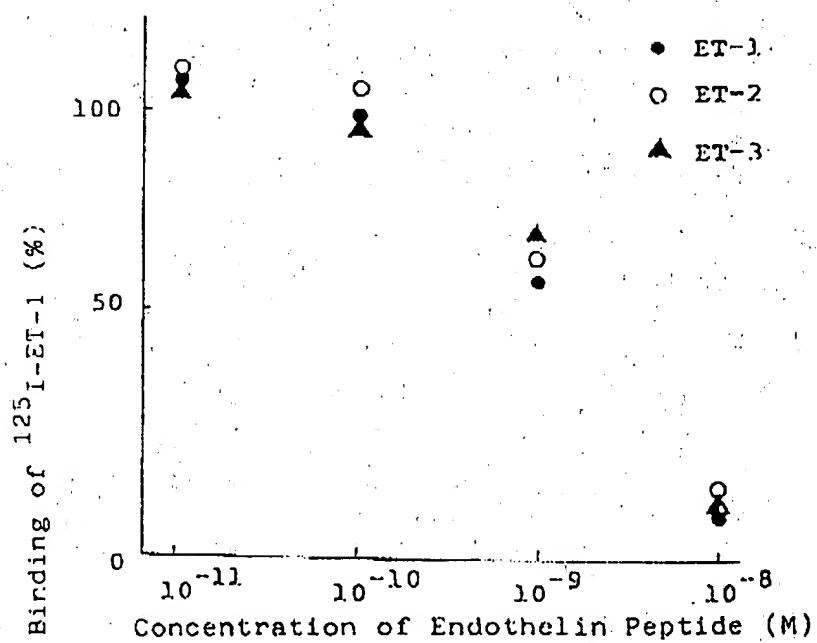
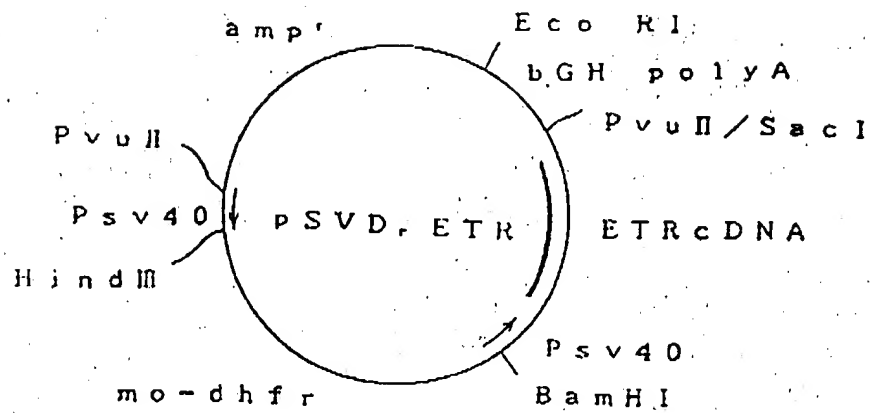


FIG. 7





European  
Patent Office

# EUROPEAN SEARCH REPORT

Application Number

EP 91 11 7144

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
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The present search report has been drawn up for all claims			
Place of search Berlin		Date of completion of search 02 December 91	Examiner JULIA P.
<p><b>CATEGORY OF CITED DOCUMENTS</b></p> <p>X: particularly relevant if taken alone Y: particularly relevant if combined with another document of the same category A: technological background O: non-written disclosure P: intermediate document T: theory or principle underlying the invention</p> <p>E: earlier patent document, but published on, or after the filing date D: document cited in the application L: document cited for other reasons &amp;: member of the same patent family, corresponding document</p>			